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Understanding the molecular basis of breast carcinoma is central to designing rational and effective therapy for this prevalent disease. We have proposed to investigate several regulatory proteins that we hypothesized may underlie at least a subset of human breast carcinomas. In investigating transcription factors of the POU domain class and their synergistic interactions with nuclear receptors, we have discovered a novel protein, expressed in the breast, that acts as a specific repressor of retinoic acid and thyroid hormone receptors. This 270 kDa protein, postulated to mediate retinoic acid and thyroid hormone receptor actions as a repressor of specific gene expression, is recruited to estrogen receptor by antagonists and its regulation appears to underlie resistance in ER positive tumors.

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5. INTRODUCTION

Breast cancer is a devastating disease, the etiology of which may be best understood by investigating the regulatory events that can maintain or restore normal growth properties. Our laboratory has investigated control of gene transcription and mammalian development, emphasizing two classes of transcription factors: POU domain factors, and nuclear receptors that mediate both positive and negative patterns of gene expression. Our ongoing studies under this Award are based on the premise that these factors, and trophic receptors under their control, are of specific significance to the etiology and treatment of breast cancer.

We have perused our original goals emphasized transcription factors of the POU domain class and their synergistic interactions with nuclear receptors, and a novel seven-transmembrane helix receptor expressed in breast. Our most important findings have been the discovery of coregulators of estrogen receptors, including novel nuclear receptor co-repressor protein, referred to as N-CoR. We have continued to emphasize the role of this factor in breast cancer, because it has important implications for the large number of women who have estrogen receptor positive tumors. A major therapeutic problem is the occurrence of resistance to the most widely used estrogen antagonist, tamoxifen or 4-hydroxy tamoxifen. We have reached an initial understanding of the mechanism of tamoxifen action which works as a repressor in normal breast and in breast tumors, and the molecular basis of the development of tamoxifen resistance. Thus N-CoR can bind to the estrogen receptor in the presence of the antagonist (partial agonist) tamoxifen, and while, in turn, is dominant to the actions of activating co-factors that associate with the receptor N-terminus. We believe that these studies will ultimately permit categorization of tamoxifen resistance and new approaches to therapy.

The morphogen retinoic acid is required for development, growth and differentiation (reviewed in 1 and 2). Retinoids, a group of analogs of vitamin A, particularly at high levels, suppress carcinogenesis in various epithelial tissues, including the mammary gland (3-6). We believe that this reflects the actions with a co-repressor that we believe may serve roles in initiation of breast cancer. The hormone effects are mediated by binding to specific nuclear receptors (3) that are members of the steroid/thyroid hormone receptor super family (4-6). This class of proteins functions as ligand dependent transcription factors that mediate the response of the hormone signal by direct control of gene expression. The estrogen and glucocorticoid receptors bind DNA as homodimers, while retinoic acid receptors preferably interact with their cognate DNA response elements as components of heterodimeric complexes (7-20) often involving a partner that our laboratory and others identified to be members of the retinoid X receptor (RXR) family (8-21). Heterodimers of retinoic acid receptor and retinoid X receptor bind with high affinity and activate transcription from response elements consisting of direct repeats, palindrome or inverted palindromic arrangements of a core recognition motif (4, 8-21). The relative orientation and spacing of the core recognition motifs play essential roles in the specificity of the DNA binding and transcriptional activation. While heterodimers of retinoic acid receptor and retinoid X receptor bind to direct repeats of core motifs spaced by 1, 2 and 5 bp (DR+1, DR+2, and DR+5), an unspaced palindrome binds to an inverted palindromic arrangement of the core motif spaced by 6-8 bp, (e.g. IP+6) reviewed in 22,23).

Recent studies indicate that heterodimeric complexes of retinoic acid receptor and retinoid X receptor molecules exhibit a polarity binding to various DNA elements (24-26), and that this polarity-specific binding may play important roles in cell-specific regulation by retinoic acid receptors. On a direct repeat spaced by 5 bp (DR+5) retinoid X receptor selectively binds to the upstream half-site and retinoic acid receptor binds to the downstream half-site (25,26). When the spacing is reduced by one base pair (DR+4 site) the element becomes a binding site for heterodimers of thyroid hormone receptor and retinoid X receptor. In this case the thyroid hormone receptor is bound to the downstream half-site and the retinoid XD receptor again interacts with the upstream half-site. However, in the case of a direct repeat spaced by 1 bp (DR+1), retinoid X binds on the 3' site (27). The stringency of this polarity-specific binding was further confirmed using specific mutants of retinoid X receptor containing the P-box residues of the glucocorticoid receptor (27). In contrast to the glucocorticoid receptor, retinoic acid receptors are not associated with heat shock proteins in the absence of hormone, but are bound to their response elements and are able to actively repress basic transcription (28,29).

The cellular thyroid hormone receptor, like the viral oncogene erbA of the Avain Erythroblastosis Virus (AEV), V-erbA, represses transcription of target genes in the absence of ligand, with hormone binding resulting in de-repression and activation (31,34,35,45). Evidence has indicated that in most cases ligand-independent repression appears to result from an active repressor function within the ligand binding domain. A ligand-independent repression function could be transferred by the carboxyl-terminal region of the thyroid hormone receptor to heterologous DNA binding domain. Fusion of the C-terminal domains of V-erbA, T3R, and RAR to the DNA binding domain of the yeast transcription factor GAL4, generated UAS-dependent transcriptional repressor proteins (31). In contrast, the RXR C-terminus fused to the GAL4 DNA binding domain did not mediate transcriptional silencing. Our laboratory and others, were able to show that this repression was mediated by the C-termini of the retinoic acid and thyroid hormone receptors (30-33), thus, thyroid hormone and with the polarity of binding on sites with asymmetric core motifs (17, 36 through 44), dictate whether the receptor exerts either positive control of gene transcription .

The molecular mechanisms responsible for nuclear receptor transcriptional silencing have not been well understood until the past year. Recent studies showed that several nuclear receptors may interact with the basal transcription factors, including TFIIß (47); however, the distal T3 receptor thyroid hormone C-terminal regions interact with TFIIß, are not sufficient to confer repression. Indeed, the regions in the hinge and N-terminal part of the ligand-binding domain of the thyroid hormone receptor are required for silencing (46,47). Co-transfection experiments suggest that these sequences which do not bind TFIIß can potentially compete for a putative soluble co-repressor molecule (47) and imply that existence of additional interaction factors required for ligand-independent repression. Under this Grant, we isolated and characterized a novel 270 kDa factor (N-CoR) characterized by an interaction domain in the distal C-terminus, and a receptor in the mutant cell and also interacts with retinoid acid receptor, but not with the unliganded estrogen, progesterone, retinoid X, glucocorticoid, or vitamin D receptors. Receptor specific mutations in this region that abolished interactions with the 270 kDa protein also eliminated the ligand-independent repression function of the thyroid hormone receptor, retinoic acid, and because 270 kDa protein can itself function as a repressor, our data suggested that the 270 kDa protein associated with the unliganded, DNA-bound thyroid hormone receptor, and retinoic acid is required for ligand-independent transcriptional repression; we, therefore, termed this protein N-CoR for nuclear receptor co-repressor (51-53).

Several lines of evidence indicate that nuclear receptors must interact with additional factors dependent on a conserved distal C-terminal motif (AF2) to mediate both activation and repression of gene expression (48-52). Biochemical assays have identified 140 and 160 kDa proteins (p140 and p160) (49,48,52) that associate with estrogen, retinoic acid, thyroid hormone, retinoid X, and potentially other nuclear receptors as the most prominent ligand-dependent putative co-activators, binding in an AF2-dependent fashion. In addition, a series of proteins exhibiting ligand-dependent interactions with the C-termini of nuclear receptors that may also function as co-activators have been identified using a yeast two-hybrid screen.

6. BODY

Over the grant, we worked to identify the p160 exhibiting ligand-dependent association with DNA-bound thyroid hormone and retinoic acid receptors in the presence of thyroid hormone and to understand how the molecules function. The ligand-binding domains of estrogen receptor and other nuclear receptors, including retinoic acid and thyroid hormone receptors, interact strongly in the cell with a conserved domain in the N-terminus of CBP and p300 in a ligand-dependent manner. Further, the putative co-activator p160 was found to interact independently and specifically with a conserved C-terminal domain in CRIB binding protein CBP and p300. Expression cloning of a family of p160 cDNAs was achieved based on estrogen receptor and CBP interaction. Several independent experimental approaches have suggested a central role of CBP in ligand-dependent activation of RAR and T3R. One of the p160 factors (NCoA-1) appears to be associated directly with liganded estrogen receptors, while a second (p/CIP) is primarily associated with CBP; yet both are required for estrogen-dependent activation. In addition, the CBP-associated factor harboring intrinsic histone acetylase function, p/CAF, is required for nuclear receptor function. Our data revealed that, in a concentration-dependent

fashion, anti-CBP IgG specifically inhibited ligand-dependent activation of transcription units containing retinoic acid response elements, without altering expression of other promoters. (57)

Because CBP and/or its related family members were required for transactivation by retinoic acid and other nuclear receptors, we investigated the possibility that putative nuclear receptor co-activators, p140 or p160, could themselves interact with CBP. A region of 105 amino acids that was sufficient for interactions with p160. In addition, ^{32}P-CBP C-terminus could detect p160 on far western experiments. These cDNAs encoding the putative p160 (nuclear receptor co-activator, NCoA) were obtained by expression cloning based on the criteria that phage plaques exhibit interaction with both CBP C-terminus and liganded nuclear receptors and gene products were identified and full copy cDNA obtained. The first (NcoA-1) encoded variant forms of the SRC-1 protein, initially reported to have a predicted molecular weight of 115 kDa (54). N-terminally extended variants included forms containing 1465 and 1402 amino acids, with predicted molecular weights of 159 and 152 kDa respectively. We also identified two additional family members (referred to as NCoA-2, and p/CIP). P/CIP proves to be very largely expressed in breast tumor cells. Based on immunodepletion experiments, these p160 factors are the biochemically-identified p160. Microinjection assays confirmed that both NCoA-1 and p/CIP are required for estrogen and retinoic acid receptor-dependent activation events.(55) Nuclear receptors, CBP, NCoA and p/CIP interact with conserved leucine charged residue rich domains (LCD) motifs, apparently amphipathic helices (i.e. hydrophobic on one gene, hydrophilic the other gene) that are present in all factors known to be recruited to the corepressor complex (57).

N-CoR AND SMRT ARE PART OF A CO-REPRESSOR COMPLEX:

Consistent with a role of N-CoR in mediating the biological effects of estrogen antagonists, decreased N-CoR levels are found to correlate with the acquisition of tamoxifen resistance by human breast cancer cell in the nude mouse model.

Recently N-CoR and SMRT have been found to be components of corepressor complexes (56,58,59) that also include the distinct histone deacetylases HDAC1 (60) and HDAC2/mRPD3 (61), as well as the corepressor mSin3 (62,63). Recent work by our laboratory and others has shown that HDAC2 is recruited to DNA via targeting by diverse site-specific transcription factors (56,58,59,64-71). This led to the hypothesis that histone deacetylation results in a structural rearrangement of chromatin and, by an as yet poorly understood mechanism, transcriptional repression. This model provides a conceptual link between external stimuli, recruitment of corepressor complexes, and resulting alterations of chromatin that modulate gene transcription.

N-CoR was independently identified as a progesterone receptor-binding protein in the obligatory presence of the antiprogestin RU486 70). Our results suggest that receptors other than the thyroid hormone and retinoic acid receptors may, under some conditions, recruit the N-CoR corepressor complex. Conversely transcriptional activation by nuclear hormone receptors, requires the histone acetylases CBP/p300 (73-73) and p/CAF (61). Thus, the ligand-mediated switch of a nuclear receptor from transcriptional repression to transcriptional activation reflects an exchange of a histone deacetylase-containing corepressor complex for a coactivation complex containing multiple histone acetylase functions (56).

This issue is clinically relevant because the development of inhibitory ligands for the nuclear receptors, that seemingly do not cause the switch in complexes, has yielded important therapeutic treatments, among them the use of tamoxifen for endocrine therapy of breast cancer. The tamoxifen-related compounds, including trans-hydroxytamoxifen (TOT) are thought to inhibit estradiol-dependent transactivation by competitive binding to the estrogen receptor (74,75). However, in certain tissues such as uterus and bone, and after long term treatment in patients with breast cancer, tamoxifen exhibits partial agonistic activity thought to be mediated by the constitutively active AF-1 domain of the estrogen receptor, although the mechanism by which tamoxifen exerts differential effects in various tissues has remained elusive (76). Substances which raise intracellular cAMP levels or stimulate the ras/MAP kinase pathway can also cause the estrogen receptor to activate in the absence of its activating ligand (77-83).

Over the past year, we have demonstrated that diverse strategies are used by the breast in regulating the association of specific N-CoR or SMRT-containing complexes with nuclear receptors, which includes in addition to the DNA site, the nature of the ligand, the levels of N-CoR/SMRT, and the action of several protein kinase-dependent signaling cascades. These diverse regulatory events alter the association of the receptor with corepressors and coactivators, and coordinately dictate whether these nuclear receptors will repress or activate the transcription of target genes. Even the region of coactivators used is altered by the signalling pathways activated in the cells (84).

The regulation of the corepressor binding appears to be biologically important for diverse functions; for example, retinoic acid receptors prove to be constitutively active in the absence of N-CoR, and the absence of either N-CoR or SMRT converts steroid receptor antagonists to functional agonists by allowing the receptor N-terminus to recruit the identical CBP/ p/CIP complex required for activation by the liganded estrogen receptor C-terminus. These regulatory events dictate the nature of the ligand response in normal and tumor cell types, and present several new approaches to problems of resistance in antagonists-treated, receptor positive breast cancers.

7. CONCLUSIONS

Modulation of the association of receptors with corepressor complex

Recent evidence has suggested that nuclear receptor function is dependent upon the recruitment of specific coactivator and corepressor complexes. In this manuscript, we present evidence that regulation of the corepressor complex is critical for normal homeostasis and that it actually serves to impose ligand-dependence on those nuclear receptors that bind N-CoR constitutively. Thus, when the binding of N-CoR to the unliganded retinoic acid receptor is prevented, the receptor becomes capable of functioning as a constitutive activator. An antiprogestin and the Class I antiestrogens (tamoxifen-like) induce recruitment of the N-CoR corepressor complex to receptors that, unlike thyroid hormone and retinoic acid receptors, do not bind effectively to corepressors in the unliganded state (51). Even pure antagonists (e.g. LG 629) of the retinoic acid receptor fail to entirely inhibit activation in the absence of N-CoR binding. Similarly, blocking receptor association with N-CoR converts the anti-estrogen TOT into an agonist, suggesting that either a decreasing level of N-CoR, or inhibition of corepressor binding to the receptor, might account for the ability of TOT to induce activation in specific cell types and we provide evidence that both types of regulation do occur. Therefore, a critical biological role of the N-CoR corepressor complex is to suppress constitutive activation and thus impart ligand dependence on the many developmental and homeostatic events controlled by specific nuclear receptors.

Our studies, in concert with reports that overexpression of N-CoR/SMRT opposes the effects of coactivators (SRC-1/NCoA-1 in particular) that allowed antagonistic ligands to act as partial agonists, indicate that the cell type-specific activation effects of tamoxifen derivatives are mediated by the receptor N-terminal (AF-1) domain and are normally blocked by the association of N-CoR with the receptor C-terminus. Thus activation function of the N-terminus appears itself to be dependent upon SRC-/NCoA-1 and the p/CIP/CBP complex. While there are weak *in vitro* interactions between the estrogen receptor N-terminus and p/CIP, as well as SRC-1/NCoA-1, it is likely that these coactivators participate in either cooperative interactions with the receptor C-terminus and/or that an additional N-terminus associated bridging factor(s) stabilizes the binding of the coactivator complex. Indeed, stimulation of cAMP-dependent and MAPK signaling pathways in cells appears to increase the interactions between the nuclear receptor AF-1 domain and components of the p/CIP/CBP complex, in parallel to facilitating the dismissal of N-CoR from the receptor AF-2 domain. We hypothesize that N-CoR or SMRT complexes on the antagonist-bound receptor C-terminus weakly interact with the constitutive receptor N-terminus and influence the association of coactivators. We suggest that, two independent activation domains in the same receptor recruit the identical coactivator complex, or a complex containing at least a subset of the factors required in ligand-induced response, and this accounts for the instances of reported synergy between those domains (85,86).

These observations support the proposal that the nature of the transcriptional response to ligand depends on the highly regulated ability of nuclear receptors to accurately shift between a coactivator complex with histone

acetylase activity and a corepressor complex containing histone deacetylase activity and provides a model system for other classes of transcription factors.

Regulation of corepressor complexes in vivo

Our biochemical data predict that any decrease in levels of N-CoR or in the affinity of the receptor for the corepressor could cause a shift in tamoxifen from antagonist to agonist, with clear implication in regard to certain pathological conditions and the use of receptor antagonists in treatment of cancers. Indeed, in mouse models of tamoxifen resistance, we observe a statistically significant correlation between decreased levels of N-CoR and the transition of MCF7-derived tumors from tamoxifen-sensitivity to tamoxifen-resistance. Additionally, many breast tumors eventually develop high levels of tyrosine kinase receptors or of intracellular cAMP (87,88) which, according to our data, would be predicted to favor the conversion of tamoxifen to agonist function by causing release of the corepressor complex. In contrast, as HepG2 cells seem to contain a normal complement of N-CoR alone, we speculate that a signaling pathway which decreases recruitment of the N-CoR component is misregulated in these cells, causing failure of TOT to act as an antagonist in the cell line. As stimulation of protein kinase A and MAP kinase pathways in the presence of estradiol results in synergistic activation of estrogen receptor in cell-type specific manner (89), these pathways could also act to amplify the agonist activity of tamoxifen even at normal levels of N-CoR, promoting tumor growth. A switch in preference for binding the coactivator or corepressor complexes provides a mechanism by which signal transduction events initiated at the cell membrane can influence diverse nuclear receptors.

This data achieved under the support of Army Grant DAMD17-94-J-4458 has therefore provided an initial insight into the molecular basis for drug resistance in receptor-function of breast cancer, and opens up an avenue to explore further advances in diagnosis and treatment of breast cancer.

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The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function

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The functionally conserved proteins CBP and p300 act in conjunction with other factors to activate transcription of DNA. A new factor, p/CIP, has been discovered that is present in the cell as a complex with CBP and is required for transcriptional activity of nuclear receptors and other CBP/p300-dependent transcription factors. The highly related nuclear-receptor co-activator protein NCoA-1 is also specifically required for ligand-dependent activation of genes by nuclear receptors. p/CIP, NCoA-1 and CBP all contain related leucine-rich charged helical interaction motifs that are required for receptor-specific mechanisms of gene activation, and allow the selective inhibition of distinct signal-transduction pathways.

CBP and p300 are functionally conserved proteins that have intrinsic acetylase activity[1,2] and are essential for the activation of transcription by a large number of regulated transcription factors, including nuclear receptors (refs 3–6), CREB (refs 7, 8), AP-1 (ref. 8), bHLH factors (ref. 9) and STATs (refs 10–12). Nuclear receptors are a large family of ligand-dependent transcription factors that bind as homodimers or heterodimers to their cognate DNA elements[13–15] and regulate genes involved in critical aspects of cell proliferation, differentiation and homeostasis. Transcriptional regulation by nuclear receptors depends primarily upon a ligand-dependent activation function, AF-2, located in the carboxy terminus and predicted to undergo an allosteric change upon ligand binding[16–22], although additional amino-terminal activation functions operate for many receptors. Consistent with this, CBP and p300 have been found to interact directly with nuclear receptors in a ligand- and AF-2-dependent manner.

In addition to CBP and p300, a series of factors that exhibit ligand- and AF-2-dependent binding to nuclear receptor C termini have been identified biochemically[3,23–25] and by expression cloning[3–6,27–32]. Two homologous factors, SRC-1/NCoA-1 and TIF-2/GRIP-1, which increase ligand-dependent transcription by several nuclear receptors in co-transfection assays[1,30,31], constitute a nuclear receptor co-activator (NCoA) gene family[1,30,31]. These findings have raised intriguing questions of whether NCOA-1, CBP or other p160 family members are required for ligand-dependent gene activation.

Here we report the cloning and characterization of a new NCoA/SRC family member, p/CIP, which complexes with a significant portion of CBP in the cell. Surprisingly, both p/CIP and NCoA-1 are required for the function of nuclear receptors, whereas p/CIP, but not NCoA-1, is required for the function of other CBP-dependent transcription factors. A series of helical leucine-charged residue-rich domains (LCDs) serve as interaction motifs within these factors and are required for the assembly of a co-activator complex and provide specificity of nuclear-receptor activation.

New members of the NCoA family

Our initial expression screening strategy for identifying members of the p160 gene family was based on the observation that the biochemically identified p160 proteins interact with a 100-amino-acid region in the C terminus of CBP (residues 2,058–2,170), as well as with the liganded oestrogen receptor[1]. This enabled us to isolate previously described NCoA-1/SRC-1 protein and a second related factor, NCoA-2 (Fig. 1a), which has a relative molecular mass of 159.6K and seems to be the murine homologue of human TIF-2 (ref. 30), part of which has been reported as GRIP-1 (ref. 31). In addition, we identified a related factor which we term p/CIP (for p300/CBP/co-integrator-associated protein) (Fig. 1a).

p/CIP is a 152K protein which is highly related to SRC-1/NCoA-1 and NCoA-2/TIF-2, having an overall amino-acid identity of 31% and 36%, respectively (Fig. 1d). p/CIP harbours a fairly well conserved N-terminal bHLH, PAS A domain (50–60% amino-acid identity), a serine/threonine-rich region, and a C-terminal glutamine-rich region, which are also present in NCoA-1 and NCoA-2. Western blot analysis indicates that p/CIP, NCoA-1 and NCoA-2 are widely expressed in adult tissues and in all cell lines tested (Fig. 1b,c).

A CBP/pCIP complex

To evaluate the association of p/CIP, NCoA-1 and NCoA-2 with CBP and nuclear receptors, a glutathione *S*-transferase fusion protein (GST) with CBP (residues 2,058–2,170) was used to affinity-purify interacting proteins from HeLa cell extract. p/CIP was consistently observed by immunoblotting using affinity-purified anti-p/CIP IgG, whereas much smaller amounts of NCoA-1 were detected by immunoblotting with anti-NCoA-1 IgG (Fig. 2a). Similarly, immunoprecipitations from whole-cell extracts using excess antiserum selective for each protein, followed by immunoblotting with anti-CBP/p300 antibody demonstrated that the vast majority of CBP/p300 co-precipitated with the new factor p/CIP, although small amounts of NCoA-1- and NCoA-2-associated CBP could be detected (Fig. 2b). Conversely, the amount of CBP/p300 remaining in the supernatant fraction following immunodepletion with anti-NCoA-1 IgG remained unchanged, but a significant fraction of CBP/p300 CBP was removed by immunodepletion with anti-p/CIP IgG (Fig. 2b). These results indicated that p/CIP could form a complex with p300/CBP in the cell.

To define the CBP-interaction domain in p/CIP, we generated deletion mutants and tested against CBP (amino acids, 2,058–2,170) by a yeast two-hybrid assay. The major CBP interaction

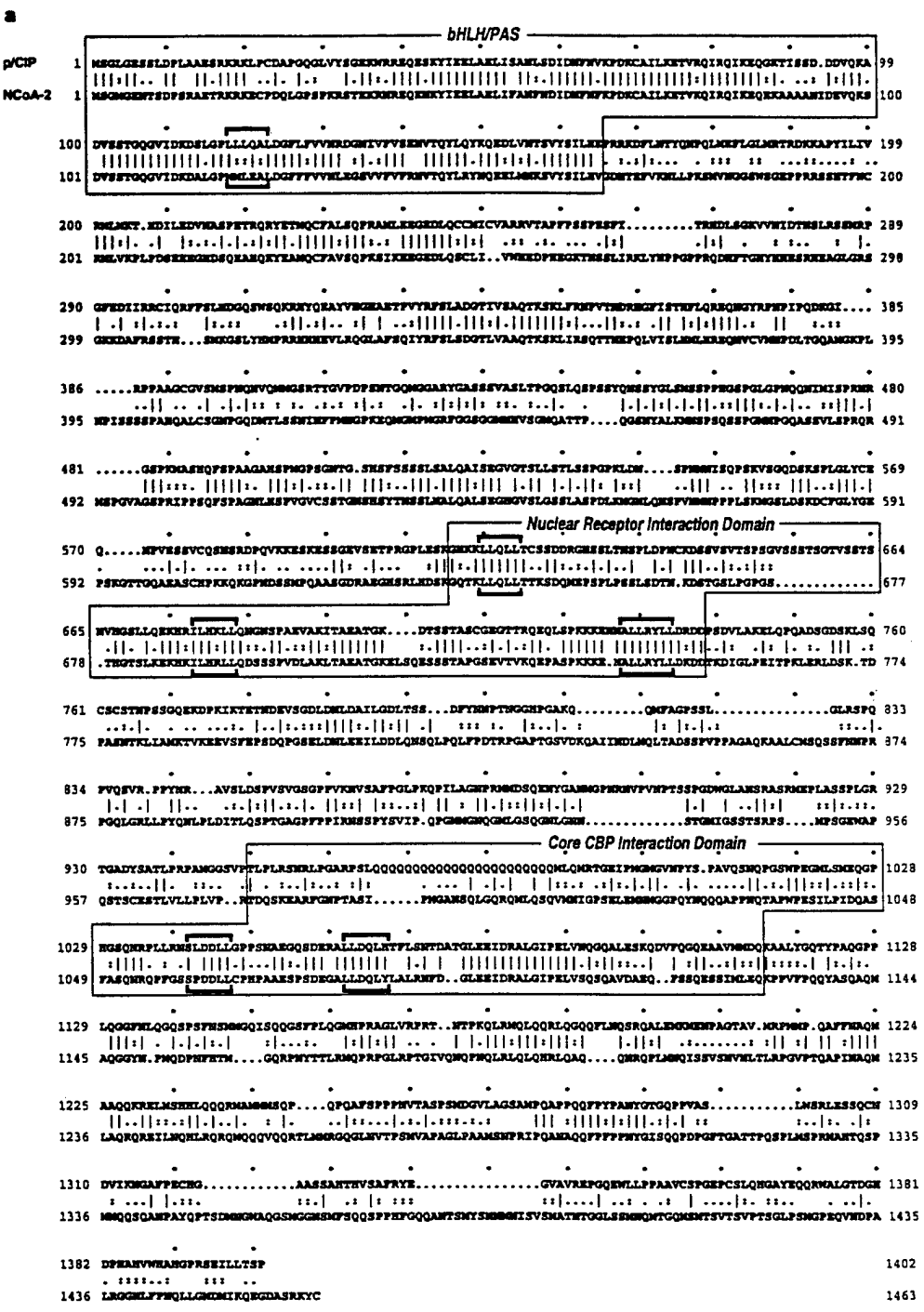

Figure 1 Characterization of a CBP-associated factor (p/CIP) and a related member of the NCoA family (NCoA-2). **a**, Comparison of the primary amino-acid sequences of p/CIP and NCoA-2. The conserved bHLH, PAS A domain, the nuclear-receptor-interaction domains and the minimal nuclear-receptor and CBP interaction domains are boxed, and repeat motifs involved in critical interactions are bracketed. **b**, **c**, Western blot analysis of total cell extracts for p/CIP, NCoA-1 and NCoA-2 in various tissues and cell lines; there is widespread expression of all three proteins, but relative levels differ. **d**, Diagram of p/CIP, showing regions of homology with NCoA-1 and NCoA-2. Asterisks refer to the repeated peptide motifs that appear to be functionally important (Figs 5, 6).

domain was located between residues 758 and 1,115, with an internal 200-amino-acid domain that could still interact. We observed a less pronounced interaction with the N-terminal region containing the PAS A domain (Fig. 2c). A single nuclear-receptor-interaction domain (residues 591–803) was localized N-terminal of the CBP/p300 interaction domain (Fig. 2c). Further mapping delineated a minimal nuclear-receptor-interaction region encompassing amino acids 680–740 in p/CIP which were sufficient for binding to the liganded nuclear receptors (data not shown). Comparable regions in NCoA-1 and NCoA-2 were found to mediate interactions with both CBP/p300 and nuclear receptors (Fig. 2d, and data not shown). GST pull-down assays of whole-cell extracts revealed that p/CIP, NCoA-1 and NCoA-2 interacted with GST fusion proteins with the oestrogen receptor (GST–ER) and the retinoic acid receptor (GST–RAR) in a ligand-dependent manner (Fig. 2e).

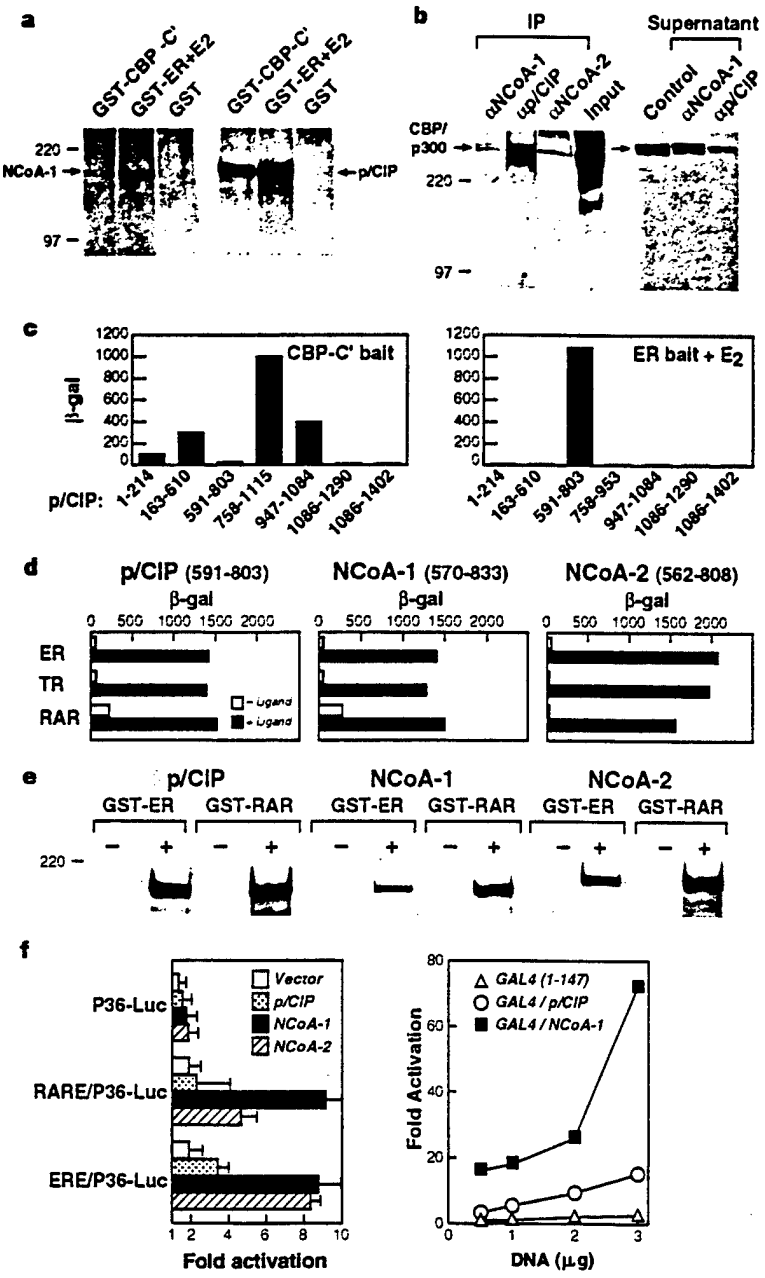

Figure 2 Biochemical analysis of p/CIP and NCoA factors. **a**, Interaction between recombinant GST proteins and NCoAs from HeLa whole-cell extracts detected using an antibody against p/CIP (left) or NCoA-1 (right). **b**, Left, co-immunoprecipitation of CBP/p300 and p/CIP. Anti-p/CIP, NCoA-1 or NCoA-2 IgG were incubated with HeLa whole-cell extracts and immunocomplexes were separated by SDS–PAGE and probed using anti-CBP/p300 IgG; right, detection of CBP/p300 in supernatant following immunodepletion of whole-cell extracts with specific anti-NCoA antibodies. **c**, Yeast two-hybrid assay mapping regions of interaction between p/CIP and CBP C terminus (amino acids 2,058–2,170) and liganded nuclear receptors (LBD). **d**, A common nuclear-receptor-interaction domain is found in p/CIP, NCoA-1 and NCoA-2 by yeast two-hybrid assays. Ligands (+) were oestradiol (10^{-6}M), Triac (10^{-6}M) and retinoic acid (10^{-6}M). **e**, p/CIP, NCoA-1 and NCoA-2 interactions with nuclear receptors *in vitro*. Recombinant GST-nuclear-receptor proteins were incubated with whole-cell extract in the presence(+) or absence (–) of ligand, and western-blotted using p/CIP-, NCoA-1- or NCoA-2-specific IgG. **f**, Reporters containing the minimal prolactin promoter (P-36 luciferase) alone or 2 copies of the indicated response elements and expression plasmids expressing p/CIP, NCoA-1 or NCoA-2 were transfected into HeLa cells in the presence of the corresponding ligand. Right, effects of varying amounts of plasmid expressing GAL4(1–147), GAL4–NCoA-1 or GAL4-p/CIP fusion proteins on a minimal $(UAS)_6$-dependent reporter.

Co-transfection with NCoA-1/SRC-1 or NCoA-2/TIF-2 expression vectors potentiated ligand-dependent activation (generally three- to eightfold), whereas co-transfection with p/CIP expression plasmids resulted in minimal or no activation effect (Fig. 2f, left). In addition, when full-length cDNAs were fused to GAL4(1–147), the activation observed by GAL–NCoA-1 was significantly stronger than GAL-p/CIP (Fig. 2f, right). Co-transfection of CBP and NCoA-1 or NCoA-2 expression vectors resulted in variable synergy (data not shown), consistent with findings reported for SRC-1 (ref. 33).

Nuclear receptors require p/CIP and NCoA-1

To investigate the function of p/CIP, NCoA-1 and NCoA-2, we used microinjection techniques with affinity-purified IgGs. Reporter genes were placed under the control of a minimal promoter containing either nuclear receptor or other response elements, as described[3]. Microinjection of anti-p/CIP IgG prevented retinoic acid from activating a retinoic-acid receptor (RAR)-dependent transcription unit (Fig. 3a), but had no effect on a promoter under the control of SP-1 elements or the cytomegalovirus (CMV) promoter. In similar experiments, we found that p/CIP was also required for the actions of oestrogen, thyroid-hormone and progesterone receptors (Fig. 3b).

To determine whether depletion of CBP, rather than p/CIP itself, was responsible for the observed effects, we next evaluated the relative abilities of p/CIP, CBP, NCoA-1 and/or NCoA-2 to rescue the inhibitory effect of anti-p/CIP IgG. No factor alone, including CBP, was able to rescue the inhibition by anti-p/CIP IgG of RAR-dependent transcription, indicating that stearic blockage or removal of CBP did not account for the observed effects. However, the simultaneous expression of both p/CIP and CBP fully restored the retinoic-acid transcriptional response in anti-p/CIP-treated cells (Fig. 3c). Therefore CBP and p/CIP are required together for nuclear-receptor activation. To confirm this strict requirement for p/CIP, the action of a 137-amino-acid region of p/CIP (residues 947–1084) containing the core CBP-interaction domain was tested by microinjection assay, resulting in complete inhibition of the retinoic-acid-dependent gene activation (Fig. 3d, left). In contrast, this fragment did not block the activity of non-CBP-dependent promoters (Fig. 3d, right).

We next investigated whether p/CIP might also be required for

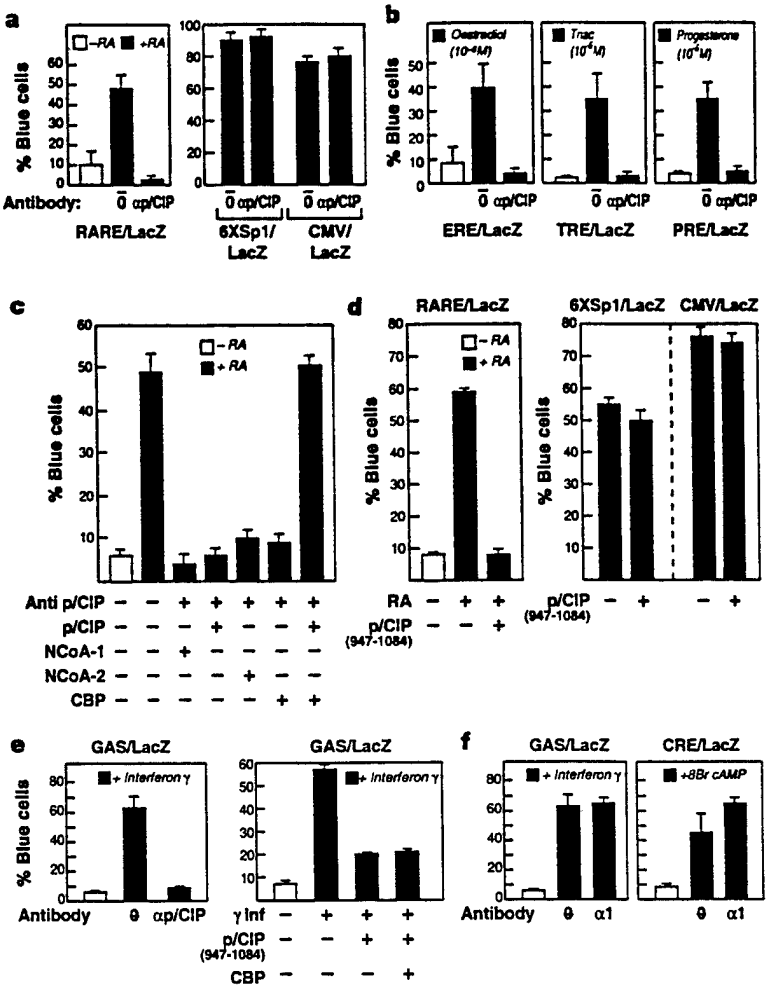

Figure 3 Role of P/CIP in function of CBP-dependent transcription factors. **a**, Effects of microinjection in Rat-1 cells of affinity-purified anti-p/CIP IgG on ligand-dependent gene activation by RAR. **b**, Similar experiments with minimal promoters with four copies of the oestrogen- (ERE), thyroid-hormone (TRE) or progesterone (PRE)-receptor response elements. **c**, Requirement for both CBP and p/CIP expression vectors to rescue anti-p/CIP IgG inhibition of RAR-dependent gene activation. **d**, Effects of expression of the p/CIP core CBP interaction domain (947-1,084) on RAR (left), and SP-1 or CMV(right)-dependent transcription. **e**, Effects of anti-p/CIP IgG (α p/CIP) on an IFN-γ-dependent promoter (GAS/LacZ)[12] (left). Effects of p/CIP (residues 947-1,084) on IFN-γ-stimulated transcriptions and failure of CBP expression vector to rescue this inhibition (right). **f**, Effects of anti-NCoA-1 IgG (α 1) on GAS and cAMP-dependent (2 × CRE) promotors. Experiments were done at least three times, with >200 cells injected; error bars are ± 2 × s.e.m.

transcriptional activation by other CBP-dependent transcription factors, such as STATs[10–12]. Therefore the effects of anti-p/CIP and NCoA-1 IgG were evaluated by immunoinjection assay in cells, initially using interferon (IFN)-γ-dependent reporters or reporters dependent on TPA (12-*O*-tetradecanoylphorbol-13-acetate). Anti-p/CIP IgG entirely inhibited STAT-dependent and TPA-dependent transcriptional activation events (Fig. 3e, and data not shown), which was not restored by overexpression of CBP alone (data not shown). Independent confirmation was provided by overexpression of the CBP-interaction domain of p/CIP (residues 947–1,084), which blocked the stimulation of transcription by interferon-γ or TPA (Fig. 3e, and data not shown). C-terminally truncated CBP failed to enhance either IFN-γ- or TPA-dependent transcription in co-transfection assays (data not shown), and could not rescue the block of retinoic-acid and IFNγ-dependent gene activation by injected anti-CBP IgG[3,12] (data not shown). These results indicate that p/CIP and CBP are a functional complex, necessary for the activity of several CBP-dependent transcription factors as well as nuclear receptors.

Roles of NCoA-1 and NCoA-2

As p/CIP is required by nuclear receptors and several CBP/p300-dependent transcription factors, we evaluated the roles of NCoA-1/SRC-1 and NCoA-2/TIF-2, which by co-transfection enhance transactivation by nuclear receptors. Microinjection of anti-NCoA-1 IgG, but not anti-NCoA-2 IgG, effectively inhibited retinoic-acid-dependent transcription (Fig. 4a), whereas these antisera failed to inhibit several control promoters lacking nuclear-receptor response elements (Fig. 4a). In addition, anti-NCoA-1 IgG fully inhibited oestrogen- and thyroid hormone-receptor stimulation (Fig. 4b) and partly inhibited progesterone-receptor stimulation (Fig. 4b). Co-injection of NCoA-1, NCoA-2 or p/CIP expression vectors revealed that the inhibitory effects of anti-NCoA-1 IgG could be reversed by either NCoA-1 or NCoA-2, but not by p/CIP (Fig. 4c), consistent with a distinct role for this factor, and in contrast to the requirement for both p/CIP and CBP to rescue inhibition by anti-p/CIP IgG. Co-injection of a CMV–CBP expression vector also failed to restore activity, consistent with the idea that both NCoA-1 and the CBP/p300/p/CIP complex are independently required for nuclear-receptor gene activation (Fig. 4c). In contrast, anti-NCoA-1 IgG exerted no effects on either cAMP- or IFNγ-dependent reporters (Fig. 3f). These observations suggest that NCoA-1 is selectively required as a co-activator for the ligand-activated nuclear receptor gene expression events, while the requirement for the p300/CBP/p/CIP complex seems to reflect a more general obligatory role in gene activation events.

Inhibition by motifs of the p/CIP complex

Because of the striking relatedness of NCoA-1 and p/CIP, despite their different function, and the activation by CBP/p300 of different classes of transcription factors, we investigated whether distinct interaction domains could selectively block the actions of specific signal-transduction pathways in the nucleus. The nuclear-receptor interaction domains of p/CIP, NCoA-1 and NCoA-2 have revealed the presence of highly conserved LCDs that share a consensus core LXXLL sequence motif (Fig. 5a). This motif is found in both the nuclear-receptor and p/CIP-interaction domains of CBP, and in the CBP-interaction domain of p/CIP. Analysis of these interaction regions by the self-optimized prediction method[34] indicates that they are helical domains, generally with amphipathic characteristics

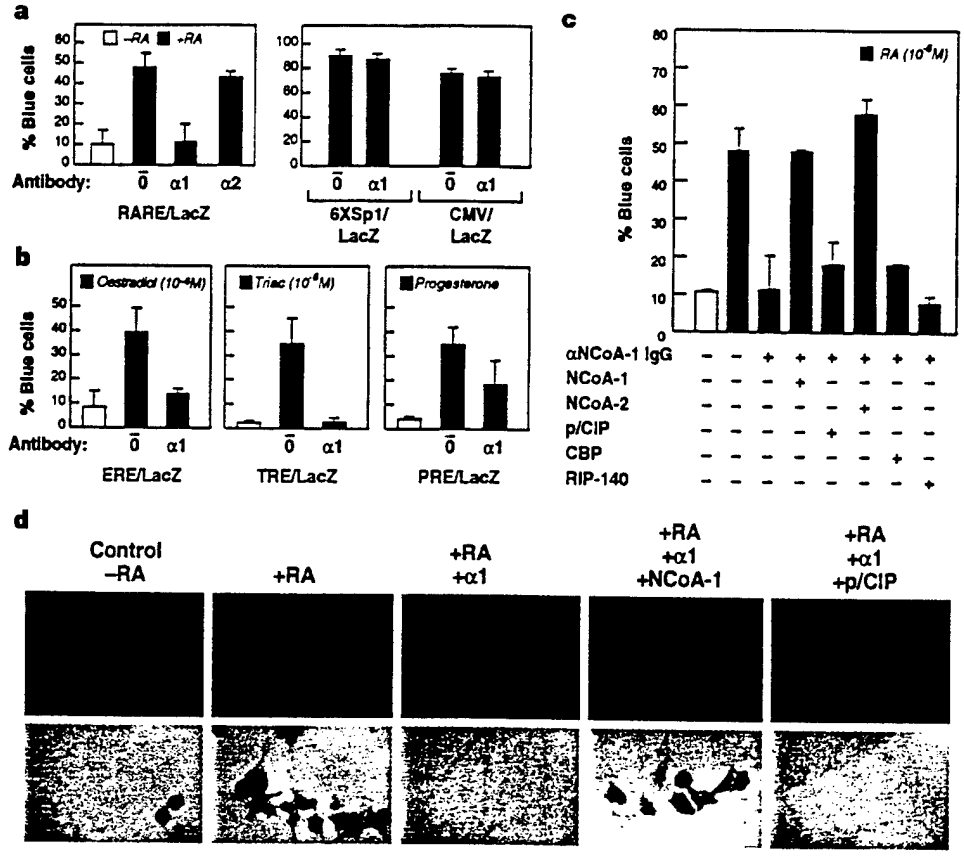

Figure 4 Role of NCoA-1 and NCoA-2 in nuclear receptor function. **a**, Microinjection of affinity-purified anti-NCoA-1, but not anti-NCoA-2 IgG blocked ligand-dependent gene activation by RAR (left) and did not inhibit expression of either the 6 × SP-1 or CMV-driven promoters (right). **b**, Results were similar using minimal promoters with two copies of the oestrogen (ERE) or T3R (TRE) response elements, with effects being less profound on progesterone(PRE)-mediated transcription. **c**, Anti-NCoA-1 IgG blocked retinoic-acid-dependent activation of the RARE/LacZ reporter, and was not rescued by CMV expression vectors expressing p/CIP or CBP; expression was fully rescued by either CMVNCoA-1 or CMVNCoA-2. **d**, Photomicrographs of rhodomine-stained injected cells and the corresponding protein of X-gal staining.

(Fig. 5a). We tested these LCDs for a critical interaction function by introducing four amino-acid mutations of this motif into the N terminus of CBP (residues 65–76), which abolishes interactions with nuclear receptors (Fig. 5b). The minimal nuclear-receptor-interaction domain of NCoA-1 contains three such helical domains, and a fourth domain (LCD6) is also present in a variant of NCoA-1 (refs 3, 29). To assess the importance of these domains in NCoA-1, a smaller region lacking helical domain 3 gave little or no decrease in binding to either oestrogen or retinoic-acid receptors; deletion of helical domain 1 gave a small but significant decrease (Fig. 5c). In contrast, a four-amino-acid substitution in the second NCoA-1 helical domain (LCD2, HRLL → AAAA), which alters the properties of this helix, abolished interaction with both oestrogen and retinoic-acid receptors. Conversely, a 37-amino-acid region of NCoA-1 containing LCD2, or a 59-amino-acid region containing LCD6, was sufficient for binding to liganded nuclear receptors (Fig. 5c, left). An excess of 24-amino-acid oligopeptide encompassing LCD2 effectively blocked interaction between liganded RAR and NCoA-1 *in vitro*, but a peptide corresponding to LCD1 was less effective. These results show that specific motifs can be both necessary and, in certain instances, sufficient for interaction.

To test for selective functional requirements of these helical motifs in the nuclear-receptor-interaction domain of NCoA-1, we generated mutations in helical domains 2 or 3 in the holoprotein, and determined whether they could rescue the anti-NCoA-1 IgG inhibition of retinoic-acid receptor. Wild-type NCoA-1 rescued activation, but a NCoA-1 holoprotein with clustered point mutations in helical domain 3 (LCD3-mut) was unable to rescue retinoic-acid receptor function. NCoA-1 containing a helical domain 2 (LCD2-mut) mutation retained some effect (Fig. 5d), consistent with the residual ability of the helical domains to mediate nuclear-receptor interactions. Surprisingly, LCD3-mut was fully functional in oestrogen-receptor-dependent gene activation, whereas LCD2-mut was now ineffective at rescuing oestrogen-receptor function (Fig. 5e). These results suggest that the helical interaction motifs of NCoA-1 afford a level of receptor specificity.

To evaluate the importance of these motifs, we tested the corresponding peptides for their ability to inhibit specific activation. NCoA-1 contains two additional related helical interaction motifs, and a peptide encompassing one of these (LCD4) can block nuclear-receptor transcription factor function and does not impair STAT function (Fig. 6a). Furthermore, a mutation in this motif impairs the function of this region in p/CIP (data not shown). Thus, specific signal transduction pathways could be selectively blocked by different helical interaction motifs.

We therefore investigated whether other motifs, not required for nuclear-receptor activation, might also be critical for co-activator function for other classes of CBP-dependent transcription factors, selectively blocking different signal-transduction pathways. Taking advantage of the fact that there is a critical STAT interaction domain within the first 100 amino acids of CBP[12], we determined whether a sequence of the CBP N-terminal 100 amino acids, distinct from the nuclear-receptor domain, might mediate interaction with STAT-1 and also be required for STAT function. We evaluated the effects of peptides corresponding to N-terminal regions of CBP on STAT-1 or retinoic-acid receptor function and found a synthetic peptide against the N-terminal 22 amino acids of CBP (CBP N'P1; Fig. 6b) that markedly inhibited IFN-γ-dependent gene activation but had no effect on the retinoic-acid receptor. The identical peptide, from which the N-terminal seven amino acids (MAENLLY) were deleted, abolished this effect (CBP N'P2; Fig. 6b), suggesting that this sequence encompassed a motif required for STAT interaction and function. Our results support the functional significance of the STAT-1 interaction motif already identified in the CBP N terminus[12].

We also tested whether the CBP N-terminal peptide could selectively block the inhibitory effects of STAT-1 or retinoic-acid-receptor-dependent transcription by evaluating its effects on stimulation by IFN-γ and retinoic acid. The simultaneous addition

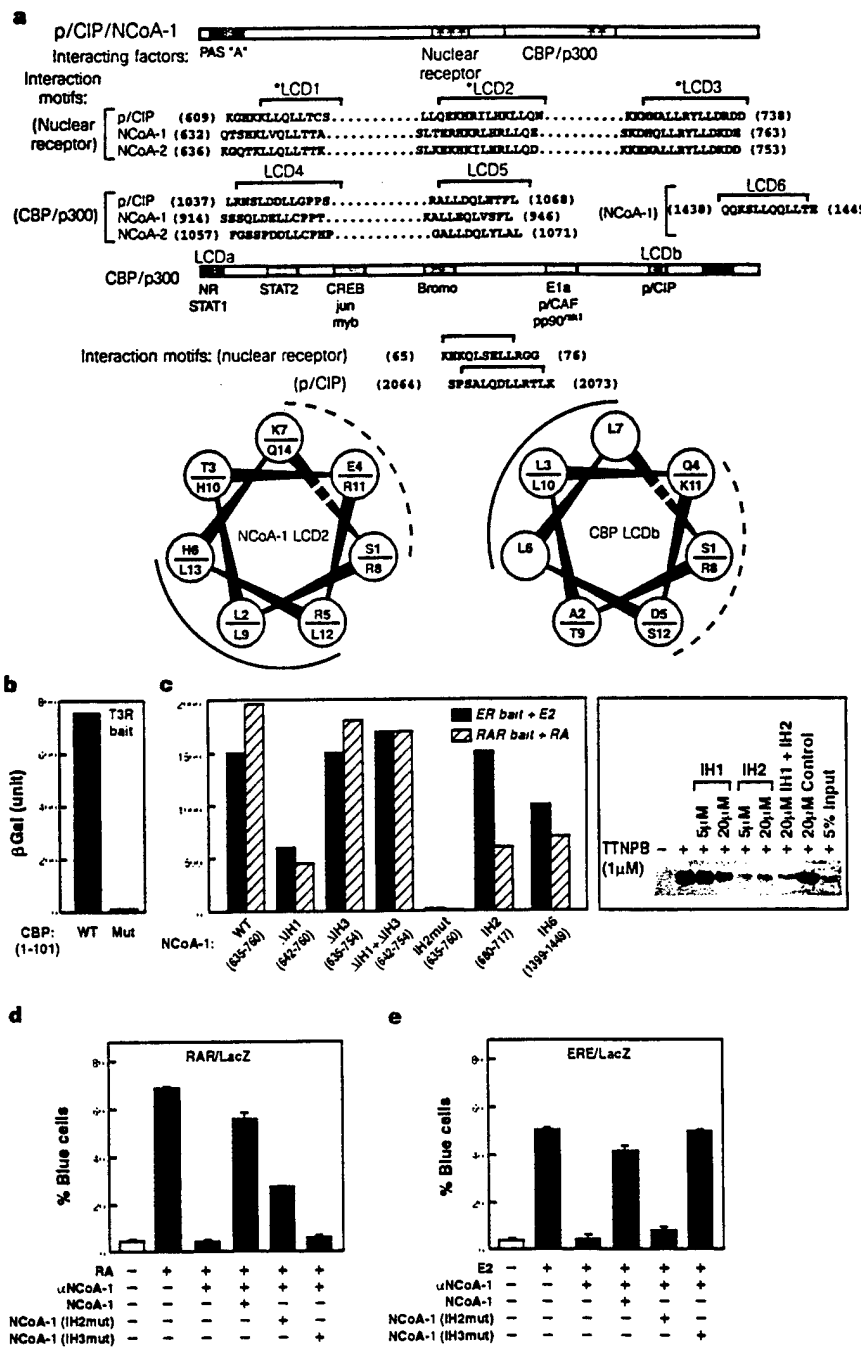

Figure 5 Leucine charged domains (LCDs) of p/CIP/NCoA/CBP. **a**, A repeated leucine-rich domain is required for protein-protein interactions between p/CIP, CBP, NCoAs and nuclear receptors. The sequences of some of these motifs are noted, with the core hexapeptide motifs indicated by brackets. Helical wheels of NCoA-1 LCD2 and CBP LCD6 are shown. **b**, Mutation of amino acids 70–73 in CBP (QLSELL→QLAAAA) resulted in a complete loss of ligand-dependent interaction with T3R. **c**, Left, assessment by the yeast two-hybrid assay of interactions between the NCoA-1 nuclear-receptor-interaction domains (residues 635–760) with nuclear receptors; centre, mutations of the LCD2 motif (RLHRLL→RLAAAA) abolished ligand-dependent interaction, whereas peptides encompassing LCD2 (37 amino acids) alone or LCD6 (59 amino acids) were sufficient for ligand-dependent interaction; right, 24-mer peptides encompassing LCD1, LCD6, or a control peptide were tested for ability to inhibit binding of ^{35}S-labelled NCoA interaction domain fragment (amino acids 635–760) to liganded RAR with TTNPB (1 μM). **d**, **e**, Functional effects of plasmids expressing mutations in LCD2 (HRLL→AAAA) and LCD3 (RYLL→AAAA) of NCoA-1 on rescue of inhibition by microinjected anti-NCoA-1 IgG (α-1) on retinoic-acid- (**d**) and oestrogen (**e**) -dependent transcription. In parts **c**–**d**, IH1, IH2 etc. represent the equivalent LCDs.

of retinoic acid and IFN-γ gave reciprocal inhibition of retinoic acid- and interferon-dependent reporter gene expression (Fig. 6c). However, the addition of CBP N′ P1 peptide relieved inhibition of RAR-dependent transcription by IFN-γ, consistent with the ideas that this inhibition represents, at least in part, competition for CBP co-activator complexes, analogous to that proposed for AP-1 and nuclear receptors[3]. Our results are consistent with the hypothesis that different 'motifs' are used in assembling CBP-dependent complexes by different classes of transcription factors and can be used to block specific signal-transduction pathways.

Discussion

Our data indicate that p/CIP, a novel factor with which a significant component of the CBP/p300 in the cell is associated, is apparently required for regulated transcription by nuclear receptors and other CBP-dependent factors, including STAT and AP-1. Our findings show that both the CBP/p/CIP complex and NCoA-1 are required to allow full ligand-activated gene transcription in the cell types that we have examined, whereas NCoA-1/SRC-1 is not required for other CBP-dependent transcription. Because CBP can associate with many additional factors, including Myb (ref. 35), YY1 (ref. 36), SREBP (ref. 37), MyoD and the HLH factors (ref. 9), p/CIP and CBP may be components of a larger complex critical for integration of several signal-transduction pathways. The existence of a co-integrator complex has obvious implications with respect to enhancer-specific functions and expanded ability to respond to diverse regulatory pathways. Whether p/CIP is required for all CBP-dependent transcription factors remains to be established. It has been shown that the N terminus of CBP alone is sufficient to potentiate CREB function in transient co-transfection assays[38,39], but the C terminus is also required in transcription assays *in vitro*[40]. Together, our findings suggest that conformational alterations in

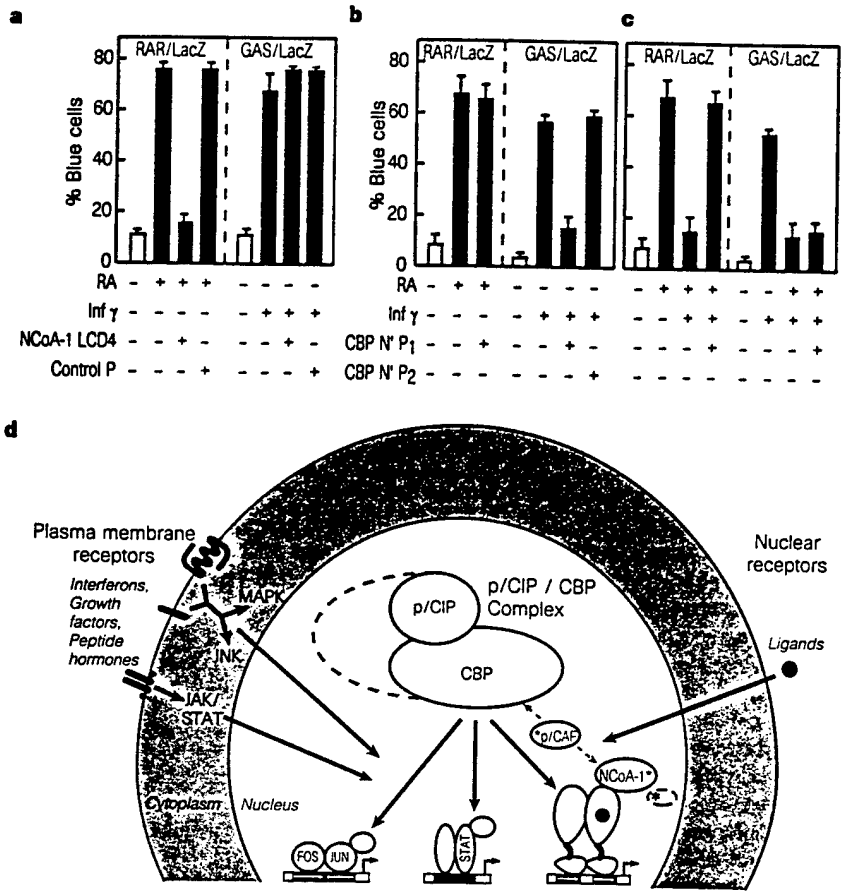

Figure 6 Distinct helical motifs block transcriptional effects of specific signal transduction pathways. **a**, Ability of a 19-mer peptide corresponding to NCoA-1 LCD4, but not a control peptide (CBP-622, control P) inhibits retinoic-acid-induced, but not IFN-γ-induced gene expression. **b**, Effects of microinjection of the N-terminal 22 amino acids of CBP (CBP N'-P1), a synthetic N-terminal CBP peptide, on retinoic-acid and interferon gene-activation events. A synthetic peptide corresponding to the identical peptide lacking the eight amino terminal amino acids (CBP N'-P2) failed to inhibit interferon-dependent gene-activation events. **c**, Similarly, IFN-γ inhibition of retinoic-acid-dependent activation of the RARE/LacZ reporter (right) was abolished by co-injection of the CBP N'-P1 peptide, which had no effect on retinoic-acid-dependent inhibition of the GAS/LacZ reporter by activated retinoic-acid reporter. **d**, Model of p/CIP/CBP (p300) function. We suggest that several signal-transduction pathways that are mediated by specific transcription factors require a functional p/CIP, CBP/p300 complex, and potentially p/CAF, with each partner being required, but not sufficient, to mediate transcriptional effects. Nuclear-receptor-specific requirements for distinct protein-protein associations through specific helical motifs (LCDs) is indicated.

CBP holoprotein, perhaps in part contributed by p/CIP, modulate interactions with transcription factors and associated regulatory proteins, including protein kinases and histone acetylases[1,2,41,42]. Furthermore, p/CAF is capable of interacting with NCoA-1 (E. Korzus and M.G.R., unpublished) as well as CBP[41], although the role of this histone acetylase in mediating transcriptional activation by nuclear receptors is unclear.

The nuclear-receptor and CBP-interaction domains within NCoA-1, NCoA-2 and p/CIP(~80–100 amino acids) contain highly related, putative helical motifs (referred to as LCDs) that are required and, at least in some cases, sufficient for interaction, but which mediate receptor-specific functions. Thus, the third helical motif in the nuclear-receptor-interaction domain of NCoA-1 is used differentially, being absolutely required for retinoic-acid function, but not for oestrogen-receptor-dependent gene activation. These data provide an insight into the molecular basis of specificity of nuclear-receptor-dependent transcriptional responses. Similar LCDs are present in CBP and in most of the factors that have been cloned based on their ability to interact with liganded nuclear receptors, including TIF-1 and RIP-140 (refs 23, 28). Thus, many factors may associate with the complexes formed on receptor homo- or heterodimers bound to their cognate DNA site and constitute an aspect of this specificity. Assembly of larger complexes of proteins, based on these interaction motifs, could provide a basis for receptor-specific and regulated aspects of nuclear-receptor function (Fig. 6d).

The finding of the similar helical interaction motifs in CBP/p/CIP/NCoAs and other nuclear-receptor-interacting factors has allowed the use of such motifs in selective blockade of gene activation events in response to specific signal-transduction pathways. Thus, peptides corresponding to CBP-interaction motifs can selectively block nuclear receptor or STAT-1 function. The actions of specific inhibitory peptides have provided additional evidence that partitioning of CBP[3,12], at least in part, accounts for *trans*-repression of nuclear receptor/STAT/AP-1 pathways. The demonstration that specific interaction motifs can selectively block gene activation by specific signal-transduction pathways has potentially intriguing applications to the study of signalling in development, as well as therapeutic implications. □

Methods

Isolation of interacting proteins. Expression cloning was done using a ^{32}P-labelled GST–CBP(2,058–2,170) or ^{32}P-labelled GST–ER ligand-binding-domain probe in the presence of 10^{-6} M oestradiol[3]. cDNAs corresponding to p/CIP, NCoA-1 and NCoA-2 were assembled into Bluescript expression vector and tested by *in vitro* translation, generating products of M_r ~160K.

Yeast two-hybrid interaction assays. The yeast strain EGY 48, the LexA-β-galactosidase reporter construct (PSH18–34) and the B42 parental vectors (PEG 202 and PJG 4-5) have all been described[3,43]. Nuclear-receptor ligand-binding domains and various CBP fragments were subcloned into PEG 202 bait vector. DNA fragments encompassing the entire p/CIP-NCoA-1 or NCoA-2 proteins were generated either by using an appropriate restriction digest or by PCR and subcloned into PJG 4-5 prey vectors. EGY 48 cells were transformed with the *lacZ* reporter plasmid pSH 18-34 with the appropriate bait and prey vectors, and plated out on Ura-His-Trp medium containing 2% galactose. Isolated yeast colonies were then allowed to grow in the same liquid medium and assayed for β-galactosidase as described[44].

Transient transfections and reporter assays. Transfection was done in either HeLa or CV-1 cells using the standard calcium phosphate procedure. Typically, 1 μg RARE- or ERE-driven luciferase reporter was co-transfected with 1 μg of the indicated vectors. The final DNA concentration was adjusted to 10 μg per 60-mm dish, incubated for 24 h, and the appropriate ligands administered for 24 h at 10^{-6} M. Alternatively, co-transfections were done using PCMX p/CIP, NCoA-1 or PCR-generated NCoA-1 fragments fused to the

GAL4 DNA-binding domain (residues 1–147). Cells were transfected with 1.0 μg $(UAS)_6$-luciferase reporter and the indicated concentrations of GAL4 fusion proteins and collected 48 h later.

Generation of affinity-purified NCoA antibodies and peptides. cDNA fragments corresponding to p/CIP(544–851) NCoA-1 (424–789), and NCoA-2(787–1,129) were subcloned into the PM vector containing an in-frame His tag and recombinant His-tagged proteins were generated and purified by nickel chelate chromatography. Purified recombinant proteins were injected into rabbits and antibodies generated and affinity-purified using standard procedures[45]. Peptide sequences, generated (Research Genetics) and confirmed by mass spectroscopy, include: NCoA-1 LCD1 (amino acids 631–647); NCoA-1 LCD2 (687–706); NCoA-1 LCD4 (907–926); CBP N'P1 (1–19); CBP N'P2(8–19).

GST-interaction assays, immunoprecipitations and enzymatic assays. Whole-cell extracts were prepared by lysing cells in NET-N buffer containing 50 mM Tris (pH 7.6), 5 mM EDTA, 0.3 M NaCl, 1 mM DTT, 0.1% NP-40 and protease inhibitors (0.2 mM PMSF, 10 μg ml^{-1} each of leupeptin, pepstatin and aprotinin), centrifuged at 30K for 1 h at 4 °C; the supernatant was stored at −80 °C.

GST–RAR(143–462), GST–ER(251–595) and GST–CBP(2,058–2,170) were generated as described[3]. 25 μl GST–Sepharose beads containing 10 μg GST recombinant proteins were incubated in the presence or absence of the appropriate ligand for 30 min at room temperature, followed by the addition of 1 mg cell extract and incubated for an additional hour at 4 °C. Complexes were then centrifuged, washed three times in NET-N buffer, separated by SDS–PAGE, and western blotted with the appropriate antibodies (1 μg ml^{-1}). For co-immunoprecipitation, 1 mg cell extract was incubated with 2 μg p/CIP or NCoA antibody for 2 h at 4 °C. Immune complexes were then precipitated with protein A–Sepharose (50% w/v). Protein complexes were separated by SDS–PAGE[46] and western blotted using 1 μg ml^{-1} of an anti-CBP/P300 monoclonal antibody (UBI). For *in vitro* competition assays, the indicated peptides were incubated with *in vitro* translated NCoA-1 before GST interaction with RAR.

Mutagenesis. Mutations in NCoA-1 and CBP were introduced by site-directed mutagenesis using a quick-change mutagenesis kit according to the manufacturer's instructions (Stratagene). Double-stranded oligonucleotides were designed so that the wild-type sequence corresponding to amino acids 695–698 and 756–759 in PCMX NCoA-1 and PJG4-5-4 NCoA-1(635–760) were substituted with alanines. A similar protocol was used to replace amino acids 70–73 in PJG4-5 CBP(1–101).

Single-cell microinjection assay. Insulin-responsive Rat-1 fibroblasts were seeded on acid-washed glass coverslips at subconfluent density and grown in MNE/F12 medium supplemented with 10% fetal bovine serum, gentacin and methotrexate. Before injection, cells were rendered quiescent by incubation in serum-free medium for 24–26 h. Plasmids were injected into the nuclei of cells at 100 μg ml^{-1}. Peptides were injected at 200 μM. Either preimmune IgG of the appropriate species, or antibodies directed against p/CIP, NCoA-1 or NCoA-2, were co-injected and the injected cell unambiguously identified. Microinjections were done using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. About 1 h after injection, cells were stimulated, where indicated, with the appropriate ligand. In rescue experiments, cells were stimulated with ligand 6 h after injection to allow protein expression. After overnight incubation, cells were fixed and stained to detect injected IgG and β-galactosidase expression[3,47]. Injected cells were identified by staining with tetramethylrhodamine-conjugated donkey anti-rabbit IgG. Data accession numbers of p/CIP and NCoA-2 sequences are AF000581 and AF000582, respectively.

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Biochemistry

Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes

(estrogen receptor/tamoxifen/corepressor complex)

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ABSTRACT **Several lines of evidence indicate that the nuclear receptor corepressor (N-CoR) complex imposes ligand dependence on transcriptional activation by the retinoic acid receptor and mediates the inhibitory effects of estrogen receptor antagonists, such as tamoxifen, suppressing a constitutive N-terminal, Creb-binding protein/coactivator complex-dependent activation domain. Functional interactions between specific receptors and N-CoR or SMRT corepressor complexes are regulated, positively or negatively, by diverse signal transduction pathways. Decreased levels of N-CoR correlate with the acquisition of tamoxifen resistance in a mouse model system for human breast cancer. Our data suggest that N-CoR- and SMRT-containing complexes act as rate-limiting components in the actions of specific nuclear receptors, and that their actions are regulated by multiple signal transduction pathways.**

Nuclear receptors are structurally related, ligand-activated regulators of a complex array of genes involved in cell proliferation, differentiation, morphogenesis, and homeostasis (1, 2). In the absence of ligand, several nuclear receptors associate with a nuclear receptor corepressor (N-CoR) (3–6) or the related factor SMRT (silencing mediator of retinoid and thyroid receptors) (7) to mediate repression. Their regulatory function is further modulated by both physiologic and pharmacologic ligands and by the actions of various signal transduction pathways that result in ligand-independent gene activation of diverse nuclear receptor family members (8–10).

N-CoR and SMRT appear to be components of cellular complexes (4, 11, 12) containing histone deacetylases (HDACs) (13, 14) and homologs of the yeast repressor Sin3 (15, 16), which are recruited to DNA via targeting by diverse DNA-binding, site-specific transcription factors (reviewed in refs. 17 and 18). Conversely, transcriptional activation by nuclear hormone receptors requires the ligand-dependent association of a coactivator complex that includes a family of nuclear receptor coactivators (NCoAs) (19–23) and also includes the histone acetylases Creb-binding protein (CBP)/p300 (24–28) and P/CAF (29, 30).

The development of inhibitory ligands for the nuclear receptors has yielded important therapeutic treatments, among them the use of tamoxifen for endocrine therapy of breast cancer (reviewed in refs. 10 and 30). However, in certain tissues such as uterus and bone, and after long-term treatment in patients with breast cancer, tamoxifen exhibits unexplained partial agonistic activity (31). Various agents that raise intracellular cAMP levels or stimulate the ras/MAP kinase pathway can similarly cause estrogen receptor (ER) activation in the presence of tamoxifen or the absence of any activating ligand (9, 32–35). In this manuscript we show that diverse molecular strategies regulate the association of N-CoR- or SMRT-containing complexes with specific nuclear receptors, including the nature of the ligand, the levels of available N-CoR/SMRT, and the action of diverse protein kinase-dependent signaling cascades, that modulate the switch from transcriptional repression to activation.

MATERIALS AND METHODS

Protein-Interaction Assays and Cell Culture. GST interaction assays and cell extracts were performed as previously described (4) with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent instead of Nonidet P-40 for ER studies. MCF-7 cells were starved overnight in medium containing charcoal-stripped serum, treated with 10^{-6} M *trans*-hydroxytamoxifen (TOT; gift of W. Lee Kraus and Benita Katzenellenbogen), 10^{-7} M 17β-estradiol, or no ligand for 4 hr, and subsequently treated with 10 μM forskolin, 0.1 mM 8-Br-cAMP, or 50 ng/ml epidermal growth factor (EGF) for 15–30 min and lysed in CHAPS. HeLa cells were incubated in stripped medium for 6 hr before transfection with 12 μg RSV-ER or RSV-ER:S118A expression plasmid/150-mm plate. Peroxisome proliferator-activated receptor γ (PPARγ) immunoprecipitations in CV-1 cells were treated for 20 min with either EGF (50–250 ng/ml final concentration), 200 μM dopamine, or 0.1 μM phorbol 12-myristate 13-acetate, and lysed in NETN (150 mM NaCl/2 mM EDTA/20 mM Tris·HCl, pH 7.4/0.5% Nonidet P-40). Detection was performed with anti-PPARγ2 (Affinity BioReagents, Neshanic Station, NJ).

Nuclear Microinjection, Staining, and Fluorescence Microscopy. Each experiment was performed on three independent coverslips totaling approximately 1,000 cells, in triplicate. Where no experimental antibody was used, preimmune rabbit or guinea pig IgG was coinjected, allowing the unambiguous identification of injected cells in addition to serving as a preimmune control. Experimental protocol was described previously (36).

Abbreviations: ER, estrogen receptor; TOT, *trans*-hydroxytamoxifen; EGF, epidermal growth factor; RAR, retinoic acid receptor; PPARγ, peroxisome proliferator-activated receptor γ; CBP, Creb-binding protein.

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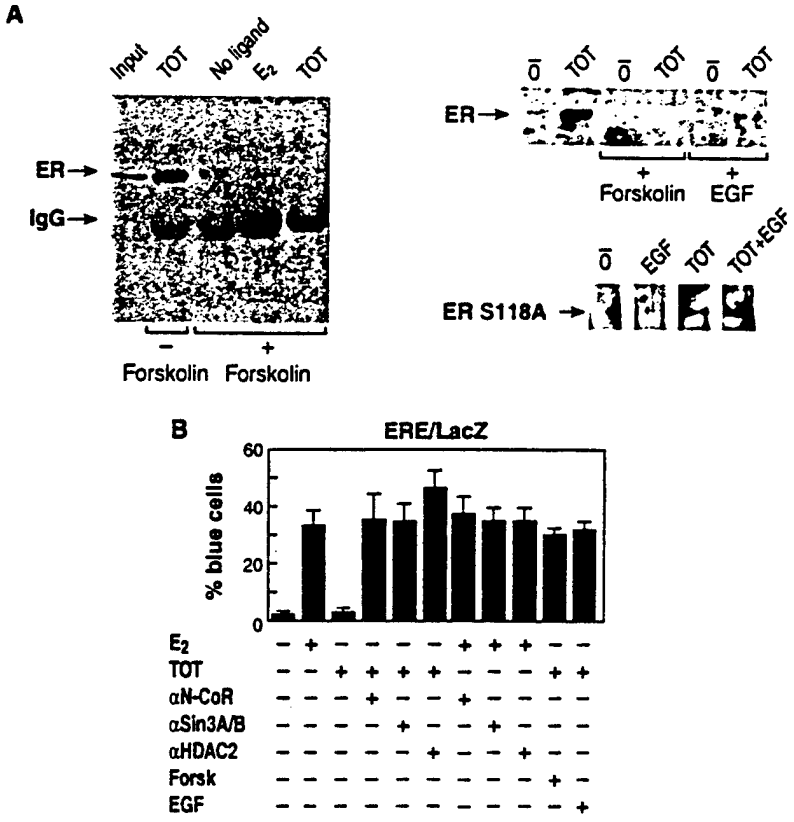

FIG. 1. (*A*) MCF-7 cells were grown in the presence or absence (Ō) of TOT and forskolin, and whole-cell extracts were immunoprecipitated with anti-N-CoR IgG and detected by using antibodies against the estrogen receptor (*Left*). ER-transfected HeLa cells were cotreated with EGF and TOT, followed by immunoprecipitation with anti-N-CoR as above. Forskolin treatment for HeLa cells is shown as a control (*Lower Right*). HeLa cells were transfected with a mutant estrogen receptor (Ser-118 → Ala), treated with the indicated combination of agents, and immunoprecipitations with N-CoR were performed as above (*Right*). (*B*) Microinjection of anti-N-CoR, anti-Sin3 (A+B), and anti-HDAC2 IgG (αN-CoR, αSin3A/B, αHDAC2) into MCF-7 cells with a LacZ reporter containing two estrogen receptor-binding elements controlling a minimal p36 promoter, in the presence or absence of 17β-estradiol or TOT. The last two columns compare these effects with those of forskolin and EGF.

Mouse Model of Tamoxifen Resistance/Tamoxifen-Stimulated Growth. MCF-7 human breast cancer cells were subcutaneously implanted into estrogen-supplemented, ovarectomized, athymic nude mice and allowed to develop as described previously (37, 38). Animal care was in accordance with institutional guidelines. Tissue was solubilized in 50 mM Tris, pH 7.8/0.2 mM EGTA/0.4 M NaCl/1 mM DTT/10% glycerol/0.1% Nonidet P-40, incubated 30 min at 4°C, and clarified by centrifugation for Western blot with anti-N-CoR (amino acids 2239–2453) using ECL (Amersham). Blots were quantitated by densitometry (Beckman DU 7 spectrophotometer) and normalized to an internal standard (MCF-7 cell extract) on each gel. Anti-actin (Chemicon) detection was performed on 1:100 dilution of extract.

RESULTS

Based on our finding that retinoic acid receptor (RAR) interacted with N-COR more strongly in the presence of antagonists (3), we tested whether ER α, which only weakly coimmunoprecipitated with N-CoR in the absence of ligand, might interact with N-CoR in the presence of an antagonist ligand. N-CoR was strongly immunoprecipitated from whole-cell extracts of MCF-7 cells (containing endogenous ER)

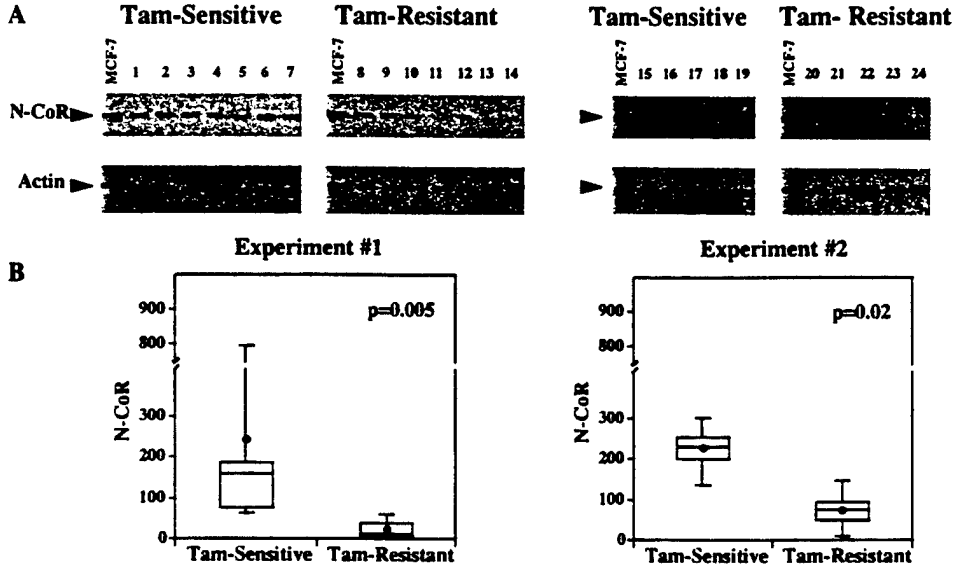

FIG. 2. A decrease in N-CoR levels correlates with the acquisition of resistance to tamoxifen by human MCF-7 breast cancer cells in a mouse model. (*A*) Western blots of whole-cell extracts from mouse tumors were normalized for total protein and probed with α-N-CoR and α-actin antibodies. Two independent sets of tumors and extracts were used. (*B*) N-CoR expression levels were quantitated by Western blot densitometry and analyzed by the Wilcoxon rank-sum test. The black circle represents the mean within each data set. The box indicates the 25th–75th percentiles; the mean (●) and median (middle line) are also shown.

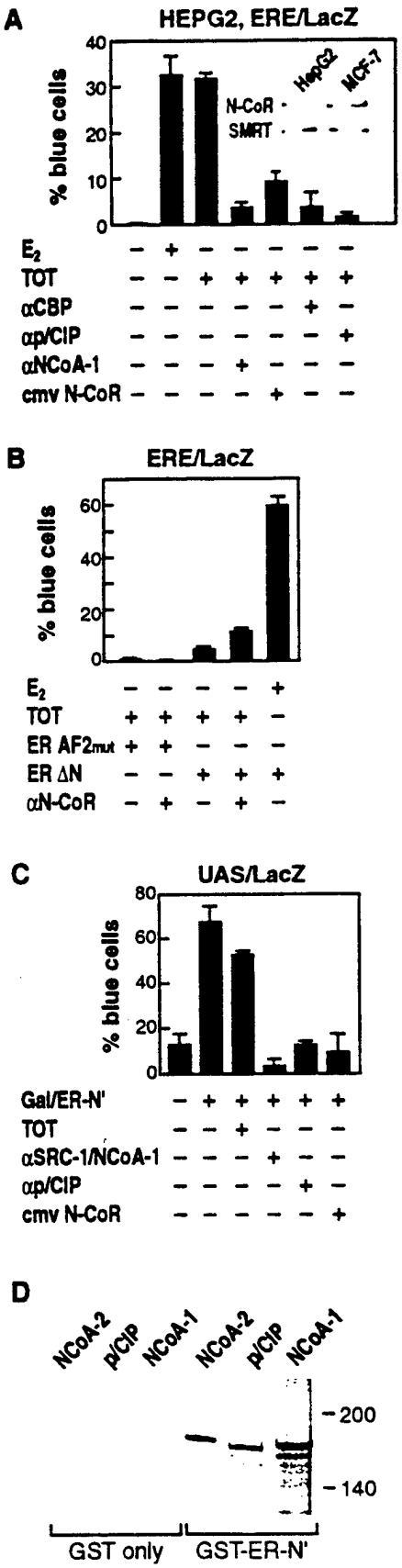

FIG. 3. TOT-bound estrogen receptor induces recruitment of the CBP coactivator complex to the N-terminal AF-1 domain. (*A*) The indicated antisera against CBP, p/CIP, or SRC-1/NCOA-1, or an expression vector for N-CoR, was microinjected as indicated into HepG2 cells with a reporter containing two estrogen response elements in front of the p36 minimal promoter. *Inset* shows Western analysis of expression levels of N-CoR and SMRT in HepG2 and MCF-7 whole-cell extract balanced for total protein loading. (*B*) Plasmids expressing mutant estrogen receptors were microinjected into Rat-1 cells with α-N-CoR IgG and a reporter containing a minimal promoter (2xEREp36LacZ). (*C*) A plasmid expressing GAL4/estrogen receptor (amino acids 1–182) was microinjected into Rat-1 cells and tested for dependence on the indicated SRC-1/NCOA-1 family members for constitutive activity. (*D*) Interaction of ^{35}S-radiolabeled proteins with GST-ER (amino acids 1–182) is shown in comparison to GST-only control lanes.

treated with the mixed anti-estrogen, TOT (Fig. 1*A*), requiring the region of N-CoR previously shown to mediate interactions with other receptors (refs. 3–7 and 39; data not shown). These ligand-specific interactions are in marked contrast to *in vitro* assays that used bacterially expressed glutathione *S*-transferase (GST) receptor and ^{35}S-labeled corepressor, in which observed ER/corepressor interactions were ligand-independent (40).

As shown in Fig. 1*A*, brief exposure of MCF7 or HeLa cells to forskolin or epidermal growth factor (EGF), agents that can switch TOT from antagonist to an agonist function (34, 41), decreased the ER/N-CoR interactions. Consistent with the observation that EGF-induced activation of the ER depends on direct phosphorylation of serine 118 (32, 35), a nonphosphorylatable mutant of ER (S118A) proved resistant to the effect of EGF on ER/N-CoR interaction (Fig. 1*A Lower Right*). Microinjection of purified IgG against N-CoR, mSin3 A/B, or HDAC2 converted TOT into an agonist in MCF-7 and Rat-1 cells (Fig. 1*B*) while exerting little effect on activity of the unliganded ER (data not shown). In the microinjection assay, treatment with forskolin or EGF also prevented the inhibitory effects of TOT.

In a mouse model system for human breast cancer that uses human MCF-7 cells growing in athymic nude mice (37, 38), prolonged treatment with tamoxifen consistently results in a transition to tamoxifen-induced tumor progression. Similarly, although tamoxifen is the most prescribed drug for the treatment of human breast cancer, all patients eventually develop drug resistance (30). Western blot densitometry analyses performed on whole-cell extracts of these tumors, normalized for total protein content and for expression levels of actin, reveal that N-CoR levels (internally normalized on each gel to identical MCF-7 control samples) declined in many of the tumors that acquired resistance to the antiproliferative effects of tamoxifen, relative to tumors retaining a response to the drug (Fig. 2*A*), whereas levels of RXR were constant between samples (data not shown). It was previously shown that the loss of tamoxifen antagonism over time is not a result of a net increase in ER levels (37). The Wilcoxon rank-sum test was used to generate a statistical summary of the results (Fig. 2*B*; $P = 0.005$ and $P = 0.02$ in two independent experiments). Because N-CoR levels were intermediate in the rapidly growing tumors from mice treated with estrogen (data not shown), the change in N-CoR protein level is not likely to be a result of cell cycle alterations accompanying the treatments.

Based on microinjection experiments using anti-CBP, p/CIP, and SRC-1/NCOA-1 IgGs, TOT-induced gene activation was found to depend on components of the coactivator complex in HepG2 cells, where TOT is a potent agonist of ER (Fig. 3*A*). Microinjection of N-CoR expression plasmid into HepG2 cells reversed TOT-induced activation, apparently overcoming an as yet unidentified mechanism that decreases binding of corepressors to TOT-bound ER.

Deletion of the N-terminal AF-1 domain of ER (ΔN75) diminished the stimulatory effect of TOT, but not 17β-estradiol, in Rat-1 cells, consistent with previously published experiments (32, 42, 43) (Fig. 3*B*), and microinjection of antibodies against N-CoR failed to activate either AF-1- or AF-2-deleted receptors (44, 45) (Fig. 3*B*). Antibodies against

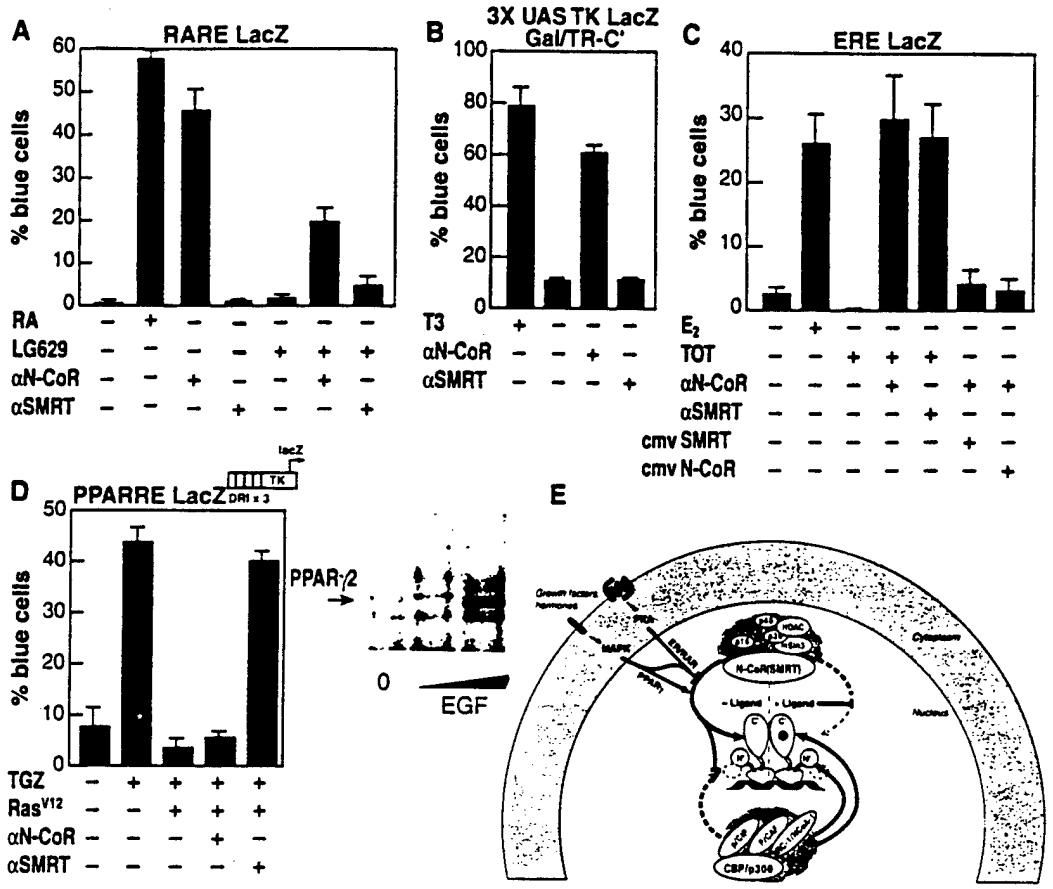

FIG. 4. N-CoR and SMRT complexes have receptor-specific roles. (*A*) A reporter under the control of retinoic acid receptor response elements was microinjected into Rat-1 cells, and effects of α-N-CoR or α-SMRT IgG were tested in the presence or absence of retinoic acid (RA) or the antagonist LG629. (*B*) A GAL fusion to the thyroid hormone receptor C terminus was microinjected, and effects of α-N-CoR or α-SMRT IgG were analyzed. (*C*) After microinjection of α-N-CoR, plasmid rescue experiments were performed as indicated. (*D*) CV-1 cells were injected with a plasmid encoding activated H-ras (val12), the indicated antisera, and a PPARγ (DR+1 site)-dependent TK promoter and treated with 1 μM troglitizone (TGZ) as indicated (*Left*). CV-1 cells were treated with increasing concentrations of EGF to stimulate MAP kinases (*Right*), and α-SMRT immunoprecipitations were performed from whole-cell extracts. Interactions were detected with antibodies against PPARγ2. (*E*) Model of regulation of nuclear receptor association with corepressor complexes. Both ligands and external signaling pathways regulate the association of specific corepressor and coactivator complexes with nuclear receptors. At least one member of a receptor homo- or heterodimer binds strongly, in the absence of ligand (or in the presence of antagonist for ER/PR), to the corepressor complex, localizing histone deacetylase activity to the promoter. This complex suppresses a constitutive N-terminal activation domain of the receptor. The corepressor complex is dismissed by agonist ligands, which allows recruitment of an acetylase-containing coactivator complex that interacts with both the receptor C-terminal AF-2 and the N-terminal AF-1 activation domains. Phosphorylation-dependent signaling pathways, initiated at the cell membrane, influence receptor activity by inhibiting the recruitment of the corepressor complex to steroid (ER/PR) and retinoid (RAR) receptors or, conversely, by stimulating its recruitment to peroxisome proliferator-activated receptor γ (PPARγ), increasing the recruitment of the coactivator complex.

the coactivators SRC-1/NCOA-1 and p/CIP abrogated the constitutive activation function of the ER N terminus, consistent with the observation that a GST/ER N-terminal fusion protein was capable of specific, but weak, interactions with SRC-1/NCOA-1, TIF2/NCOA-2, and p/CIP (Fig. 3 *C* and *D*) and a weak interaction between *in vitro* translated N-CoR and the ER N terminus.

Receptor-specific effects of N-CoR or SMRT could be demonstrated by using antibodies exhibiting no detectable cross-reactivity. Anti-N-CoR IgG, but not anti-SMRT IgG, relieved both retinoic acid and thyroid hormone receptors (Fig. 4 *A* and *B*). Additionally, anti-N-CoR IgG resulted in constitutive activation by RAR that apparently utilized the p/CIP/CBP complex required for ligand-dependent activation, as it was prevented by a p/CIP domain (amino acids 947-1084) that blocks CBP-dependent transactivation (22) (Fig. 4*A*). Anti-N-CoR was also capable of converting the RAR antagonist LG629 into a weak agonist (Fig. 4*A*). Repression by TOT-bound ER (Fig. 4*C*) and RU486-bound progesterone receptor (data not shown) was reversed by either anti-N-CoR or anti-SMRT IgG, thus converting antagonists to agonists, with either N-CoR or SMRT plasmid capable of reversing the effect of anti-N-CoR IgG (Fig. 4*C*). In contrast, PPARγ-mediated repression, stimulated by activation of the MAP kinase cascade (46), was blocked only by microinjection of antisera against SMRT (Fig. 4*D*), consistent with the observations that SMRT bound PPARγ on DNA (47) and that EGF enhanced binding of PPARγ to SMRT in whole-cell extract from EGF-treated CV-1 cells that contain endogenous PPARγ (Fig. 4*D*).

DISCUSSION

These studies reveal that the corepressor complex actually serves to impose ligand dependence on RAR, and that the anti-estrogen TOT is converted into an agonist by anti-N-CoR IgG. This suggests that either a decreasing level of N-CoR, or inhibition of corepressor binding to the receptor, might account for the ability of TOT to induce activation in specific cell types and in late-stage cancers of the breast. We have provided

evidence that both types of regulation occur *in vivo*. Activation by tamoxifen derivatives is mediated by the ER N-terminal (AF-1) domain, the function of which appears to depend on SRC-1/NCOA-1 and the p/CIP/CBP complex. In the case of ER and progesterone receptor (PR), we hypothesize that both N-CoR and SMRT complexes bind to the antagonist-bound ER C terminus and interact weakly with the constitutive ER N terminus, preventing the association of the coactivator complex. In contrast, RAR and TR appear to preferentially require N-CoR, and PPARγ selectively utilizes SMRT, perhaps reflecting preferences that are DNA- and receptor-dependent. ER and PR appear to utilize and require both corepressors in the presence of antagonists. Our observations support the model that the nature of transcriptional response to specific ligands depends on the ability of diverse signal transduction pathways to modulate the switch in nuclear receptors between a coactivator complex with histone acetylase activity and a corepressor complex with histone deacetylase activity (4, 12, 22) (Fig. 4*E*). The rate-limiting requirement of N-CoR/SMRT in estrogen receptor function suggests that there is a critical intracellular balance between the levels of N-CoR/SMRT and CBP/p300.

The data predict that a decrease in levels of N-CoR or in the affinity of the receptor for the corepressor could cause a shift in tamoxifen from antagonist to agonist, with clear implications for the use of receptor antagonists in treatment of cancers. Tamoxifen resistance (30, 31) may also be produced by decreased levels of N-CoR, overactivation of tyrosine kinase receptors or of protein kinase A (48), or by an unidentified titratable signaling pathway that regulates recruitment of N-CoR, as occurs in HepG2 cells. Because both the N-CoR corepressor complex, which plays a role in repression of transcription factors other than nuclear receptors (refs. 4, 11, and 49; R.M.L. and M.G.R., unpublished data; S. Hiebert, personal communication), and the CBP coactivator complex appear to be rate-limiting (21, 22), the regulated switch in their association that we have documented for nuclear receptors is likely to be prototypic for gene activation and repression events by many classes of transcription factors.

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Mediation of Receptor-Modulated Gene Expression

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ABSTRACT

Recent data support a model predicting that the ligand-induced switch of nuclear receptors and other classes of transcription factors from repression to activation functions reflect, in part, the exchange of complexes containing histone deacetylases with those containing histone acetylase activity. One corepressor complex contains N-Cor and mSin3 A/B as well as the histone deacetylase mRPD3, and additional components, including a p30 component that constitutes a complex that is required for repression not only by nuclear receptors and Mad but also functions on other classes of transcription factors, including homeodomain and POU domain factors. In contrast, an activation involves the actions of a series of factors, which include CBP/p300 related SRC/NCoA factors, P/CAF, and P/CIP. Different classes of mammalian transcription factors, including nuclear receptors, CREB, STAT-1, and a POU domain factors, appear to functionally require distinct components of the putative coactivator complex, and distinct domains of these factors appear to be required, indicating transcription factor-specific differences in configuration and content of the co-activator complex, and both distinct requirements for specific acetyltransferase activities. The conformational specificity of the coactivator complex is modulated by different signalling pathways. However, this coactivator complex is one of a series of potential critical nuclear receptor associated complexes. To begin to understand this multiplicity of factors, we have investigated the association of coactivator complexes with specific DNA-bound transcription factors, finding they are highly regulated, linking the actions of signal transduction pathways to ligand-dependent gene activation events. Coactivator, receptor action is based upon a specific recognition code, regulated by LXXLL motifs. The requirements and structural basis of these associations based on co-crystal structure permits predictions about receptor homodimer and heterodimer function, selection of coactivator complexes, and biochemical interactions between receptor heterodimers, which will be discussed.

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12. PERSONNEL

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